



TITLE:

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CITATION:

Nagatomo, F ...[et al]. Effects of exposure to hyperbaric oxygen on oxidative stress in rats with type II collagen-induced arthritis.. Clinical and experimental medicine 2010, 10(1): 7-13

ISSUE DATE:

2010-03

URL:

<http://hdl.handle.net/2433/97894>

RIGHT:

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Clinical and Experimental Medicine (Original Article)

Effects of exposure to hyperbaric oxygen on oxidative stress in rats with type II collagen-induced arthritis

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Abstract Arthritis was induced in 9-week-old female Dark Agouti rats by injecting type II collagen. Serum levels of the derivatives of reactive oxygen metabolites (dROMs), which are oxidative stress markers, and C-reactive protein (CRP) in arthritic rats that were exposed to a pressure of 1.25 atmospheres absolute and an oxygen concentration of 36% for 3 weeks (arthritis + HBO group) were compared to those of control rats (control group) and arthritic rats that were not exposed to hyperbaric oxygen (arthritis group). The body weights of the arthritis and arthritis + HBO groups were lower than that of the control group, whereas no difference in the body weight was observed between the arthritis and arthritis + HBO groups. The serum levels of dROMs and CRP in the arthritis group were higher than those in the control and arthritis + HBO groups. No difference in the serum level of CRP was observed between the control and arthritis + HBO groups. These results indicate that the conditions of hyperbaric oxygen exposure used in this study are effective for reducing the levels of reactive oxygen species, which are overproduced during arthritis.

Key words: C-reactive protein; derivatives of reactive oxygen metabolites; hyperbaric oxygen exposure; rat; reactive oxygen species; type II collagen-induced arthritis

Introduction

Highly reactive and toxic transient chemical species, referred to as reactive oxygen species (ROS), are overproduced by phagocytes during the development and progression of inflammatory processes and pathogenesis in a number of diseases [1]. The excessive production of ROS can damage cellular components such as nucleic acids, lipids, proteins, membranes, and matrix components. In addition, ROS serve as important intracellular signaling and regulatory molecules that enhance the synovial inflammatory-proliferative response [2]. Oxygen metabolism plays an important role in the pathogenesis of many joint diseases. The potential sources of ROS production are numerous in the case of joint diseases. In degenerative joint diseases, proinflammatory factors such as cytokines and prostaglandins are released at sites of inflammation and destruction together with ROS [3]. The production of tumor necrosis factor (TNF)- α contributes to excessive ROS release and causes substantial reduction in the activity of antioxidant enzymes in inflammatory joints. Several other proinflammatory cytokines, including interleukin (IL)-1, IL-6, and IL-8, are produced by synovial cells; these cytokines recruit neutrophils into the synovial fluids and exert their effects by activating the intracellular signaling pathways [4]. C-reactive protein (CRP) is a marker of inflammation and destruction of tissues such as cartilage and bone. The serum level of CRP correlates with the production of derivatives of reactive oxygen metabolites (dROMs), which are oxidative stress markers [5, 6].

An elevation in atmospheric pressure accompanied by an increase in oxygen concentration enhances the partial pressure of oxygen and increases the concentration of dissolved oxygen in the plasma. We determined that a

pressure of 1.25 atmospheres absolute (ATA) and an oxygen concentration of 36% are required for obtaining effective responses with regard to oxidative metabolism [7, 8]. Previous studies [9, 10] have reported low levels of partial pressure of oxygen in the synovial fluid of arthritic joints. These joints are characterized by hypoxia, which is caused by increased oxygen demand and decreased blood flow [11–13]. Hypoxic conditions induce the production of proinflammatory cytokines and excessive ROS release in inflammatory joints. We hypothesized that increased pressure and enhanced delivery and uptake of oxygen induced by exposure to hyperbaric oxygen are effective in reducing arthritis-induced inflammation because exposure to hyperbaric oxygen may prevent and improve hypoxic conditions in inflammatory joints.

In this study, we compared the serum levels of dROMs, TNF- α , IL-6, and CRP in arthritic rats that were exposed to hyperbaric oxygen to those of control rats and arthritic rats that were not exposed to hyperbaric oxygen. We verified that the conditions of hyperbaric oxygen exposure used in this study are effective for decreasing the levels of ROS, which are overproduced during arthritis.

Materials and methods

All experimental procedures and animal care were conducted in accordance with the guidelines stated in the Guide for the Care and Use of Laboratory Animals issued by the Institutional Animal Experimentation Committee of Kyoto University.

Experimental animals

Bovine type II collagen (CII) was dissolved in 0.1 M acetic acid at a concentration of 2 mg/ml by stirring overnight at 4°C. Dissolved CII was frozen at a temperature of -70°C until use. We immunized 9-week-old female Dark Agouti (DA) rats (n = 12), which are susceptible to induction of adjuvant arthritis, with an emulsion of 2 mg/ml of CII in incomplete Freund's adjuvant (IFA). The emulsions were prepared by homogenizing 1 part of dissolved CII into 1 part of IFA placed in an ice-water bath. The rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight); thereafter, 0.2 ml of the emulsion was intradermally injected into 3–5 other sites located on the back of each rat. Following injection, half of these rats (arthritis + HBO group; n = 6) were exposed to a pressure of 1.25 ATA (950 mmHg) and an oxygen concentration of 36%, automatically maintained by a computer-assisted system, for 3 weeks, while the other rats (arthritis group; n = 6) were placed in a hyperbaric chamber under normal conditions (1 ATA (760 mmHg) and 21% oxygen concentration); therefore, this group was not exposed to hyperbaric oxygen. Nonimmunized DA rats (control group; n = 6) served as controls. All rats were individually housed in same-sized cages in a room maintained under controlled 12-h light-dark cycles (lights switched off from 2000 to 0800) at a temperature of $22 \pm 2^\circ\text{C}$ with a relative humidity of 45–65%. Food and water were provided *ad libitum* to all groups. The body weight of each rat was measured every week. The pictures of the paws in all rats were taken when they were 12 weeks old. The arthritis severity was determined by means of visual examination, on the basis of the number of instances of knuckle swelling (0 point, no sign; 1 point, 1 or 2 digits; 2 points, 3 digits; 3 points, 4 digits; and 4 points, all digits), and the degree of arch edema (0 point, no sign; 1

point, light; 2 points, mild; 3 points, heavy; and 4 points, severe). The arthritis score of each rat was calculated by determining the average of the points assigned to individual paws. Therefore, the lowest arthritis score corresponded to 0 points, while the highest arthritis score corresponded to 8 points.

Blood sampling and biochemical measurements

Following 12 h of fasting, blood samples were collected from the abdominal aorta of the rats anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). The blood samples were centrifuged (6,000 *g*; 1 min) and evaluated photometrically. We used a device capable of determining the levels of free radicals and antioxidant potentials (Free Radical Analytical System 4; Health & Diagnostics; Grosseto, Italy) to measure the serum level of dROMs [14]. The serum level of dROMs was used as an index to determine the level of oxidative stress induced by measuring the amount of organic hydroperoxide (ROOH) converted into radicals that oxidize N,N-diethyl-para-phenylenediamine. The serum levels of TNF- α (R&D Systems, Inc.; MN, USA), IL-6 (R&D Systems, Inc.), and CRP (Helica Biosystems, Inc.; CA, USA) were measured by a routine laboratory method using the enzyme-linked immunosorbent assay (ELISA) kit.

Statistical analysis

Values are presented as mean and standard deviation. One-way analysis of variance (ANOVA) was used to evaluate the differences among groups. When the differences were found to be significant, further comparisons were made by performing *post hoc* tests. A probability level of 0.05 was

considered to be statistically significant.

Results

Body weight

No difference in the body weight was observed among the control, arthritis, and arthritis + HBO groups at 9, 10, and 11 weeks of age (Figure 1). In contrast, the body weights of the 12-week-old arthritis and arthritis + HBO groups were lower than that of the age-matched control group.

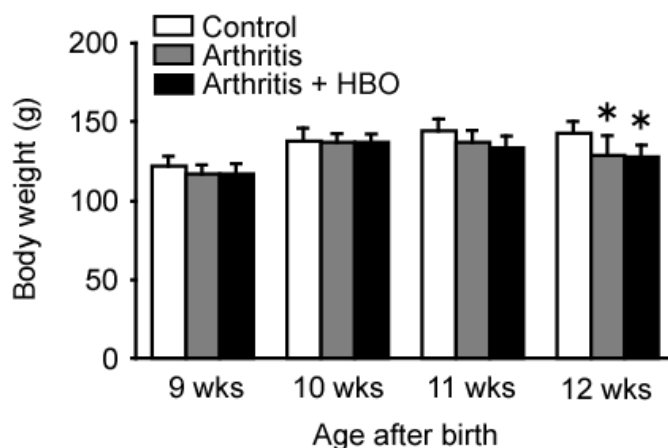


Fig. 1 The body weights of control rats and arthritic rats that were and were not exposed to hyperbaric oxygen. Data are presented as mean and standard deviation as determined from 6 animals. HBO, hyperbaric oxygen exposure. * $P < 0.05$ compared to age-matched control rats.

Arthritis severity

The rats in the control group were not arthritic (Fig. 2, A1–6). In contrast, the rats in the arthritis (Fig. 2, B1–6) and arthritis + HBO (Fig. 2, C1–6) groups presented with knuckle swelling and/or arch edema. No difference in

the arthritis score was observed between the arthritis (6.0 ± 2.3 , $n = 6$) and arthritis + HBO (4.8 ± 1.6 , $n = 6$) groups.

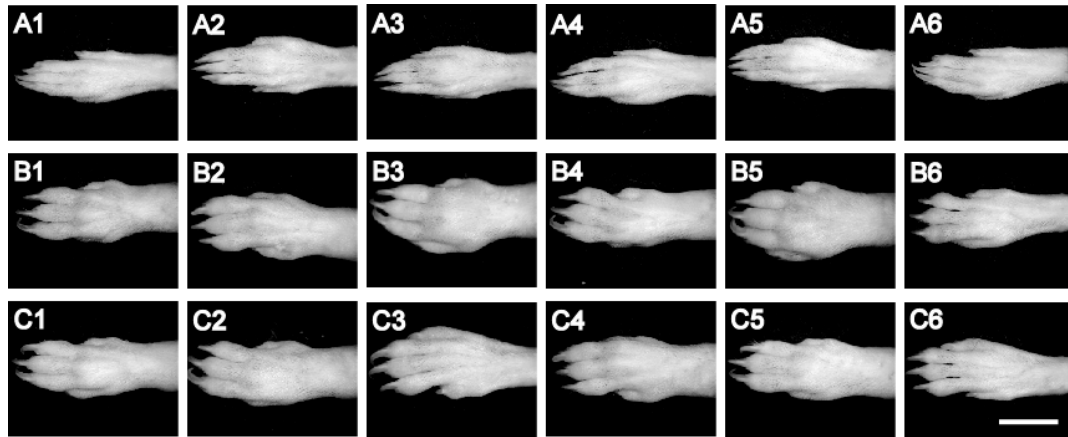


Fig. 2 The hind-paws of control (A1–6) and arthritic rats that were (C1–6) and were not (B1–6) exposed to hyperbaric oxygen. The scale bar on C6 indicates 1 cm.

Serum level of dROMs

The serum levels of dROMs in the arthritis and arthritis + HBO groups were higher than that in the control group (Figure 3A). The serum level of dROMs in the arthritis + HBO group was lower than that in the arthritis group.

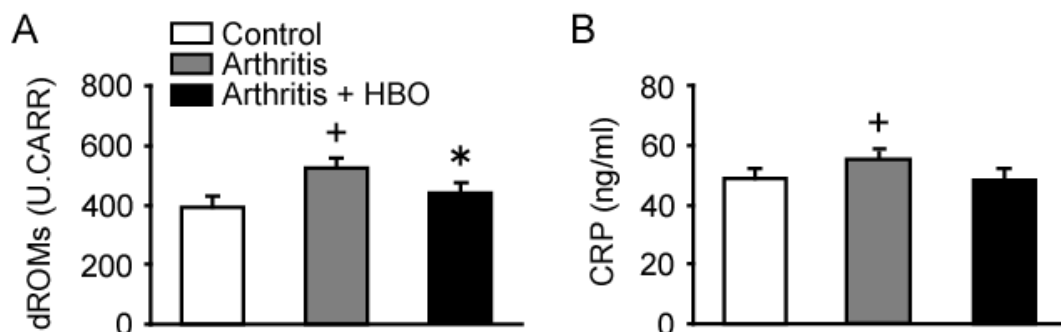


Fig. 3 The serum levels of derivatives of reactive oxygen metabolites (A) and C-reactive proteins (B) in control rats and arthritic rats that were and were not exposed to

hyperbaric oxygen. Data are presented as mean and standard deviation as determined from 6 animals. dROMs, derivatives of reactive oxygen metabolites; U.CARR, unit of Carratelli, which is a conventional unit named after an Italian biologist who developed a scale based on the observations from a group of more than 5,000 non-smoking healthy subjects with age ranging from 14 to 80 years (1 U.CARR = 0.08 mg of hydroperoxide/100 ml hydrogen peroxide); CRP, C-reactive protein; HBO, hyperbaric oxygen exposure. $*P < 0.05$ compared to control rats; $^+P < 0.05$ compared to control and arthritis + HBO rats.

Serum levels of TNF- α , IL-6, and CRP

Neither TNF- α nor IL-6 was detected in the rats, irrespective of whether they were arthritic and were exposed to hyperbaric oxygen. The serum level of CRP in the arthritis group was higher than those in the control and arthritis + HBO groups (Figure 3B). No difference in the serum level of CRP was observed between the control and arthritis + HBO groups.

Discussion

Type II collagen-induced arthritis

Mice and rats with type II collagen-induced arthritis are widely used as experimental animal models of inflammatory polyarthritis with clinical and pathological features similar to those of rheumatism [15, 16]. The DA rats are susceptible to induction of adjuvant arthritis. Arthritis is observed in 74% and 100% DA rats at 2 weeks and 3 weeks after immunization, respectively (SLC Inc.; Tokyo, Japan). The body weights of DA rats are observed to gradually decrease following immunization; the body weights are the lowest at 3 weeks

after immunization and subsequently recover to the control level (SLC Inc.). In this study, we examined certain parameters of DA rats at 3 weeks after immunization because all immunized rats demonstrated high severity of arthritis, including the signs of knuckle swelling and/or arch edema (Fig. 2, B1–6). These arthritic rats may undergo further changes leading to cartilage and bone destruction when studied for a prolonged period; similar results were observed in a previous study using mice with type II collagen-induced arthritis [17].

Serum level of dROMs in arthritic rats

Oxidative stress is the condition in which the production of oxidants exceeds the capacity to neutralize them. Several factors could be involved in the generation of oxidative stress in inflammatory joints [18, 19]. ROS are produced during many metabolic processes, including mitochondrial respiration and enzyme activities. ROS concentrations are regulated by maintaining the balance between their production and elimination by antioxidants. An appropriate balance is crucial for normal cell and tissue function. In contrast, the excessive production of ROS damages cellular components, including nucleic acids, lipids, proteins, membranes, and constituents of the extracellular matrix such as proteoglycans and collagens [2]. Previous studies [20, 21] have reported that excessive production of ROS causes an accelerated damage to joint cartilage and osteoclast activation. We observed that the serum levels of dROMs, which serve as a marker of oxidative stress, were higher in arthritic rats than in control ones (Fig. 3A); this finding suggests that arthritis induces an excessive production of ROS.

Serum levels of TNF- α , IL-6, and CRP in arthritic rats

Several proinflammatory cytokines (TNF- α , IL-1, IL-6, and IL-8) are produced by synovial cells and recruit neutrophils to the synovial fluids in arthritic rats. However, we detected neither TNF- α nor IL-6 in the DA rats with arthritis. Previous studies [22, 23] have reported increased levels of TNF- α and IL-1 β in Wistar or Lewis rats with type II collagen-induced arthritis. We were unable to elucidate the reason underlying the inability to detect cytokines in arthritic DA rats.

We observed increased serum levels of CRP in arthritic rats (Fig. 3B). Previous studies [24, 25] have reported that the serum level of CRP correlated with that of proinflammatory cytokines such as IL-6. Increased serum levels of CRP are observed in inflammatory diseases [26]. Therefore, the increased serum levels of CRP reflect the arthritis severity, including signs of knuckle swelling and/or arch edema (Fig. 2, B1–6).

Major drugs for arthritis

It is widely accepted that reduction of oxidative stress by superoxide dismutase (SOD) affects the course of inflammation. Several studies [27–30] have focused on synthetic low-molecular weight compounds that mimic the effects of SOD. Among the various families of SOD mimetics, the most promising are nitroxides (tempol) and Mn (II) pentaazamacrocyclic ligand (M40403). Tempol diminishes hydroxyl radical production and decreases the cytotoxic effects of hydrogen peroxide and peroxynitrite [27]. Furthermore, it decreases inflammation and tissue damage in rats with type II collagen-induced arthritis. M40403 decreases the release of proinflammatory cytokines such as

TNF- α , probably by inhibiting the expression of the transcription factor, nuclear factor (NF)- κ B [28]. The beneficial effects of M40403 have been reported in rats with collagen-induced arthritis [29, 30].

Treatment with 3,7,11,15-tetramethyl-2-hexadecene-1-ol (phytol) increases oxidative burst *in vivo*, decreases the autoimmune response, and ameliorates both the acute and chronic phase of arthritis in rats [31]. Furthermore, treatment with alpha-lipoic acid (LA), which is a co-factor for mitochondrial α -keto dehydrogenase complexes and which participates in S-O transfer reactions, suppresses the development of collagen-induced arthritis in mice. A previous study [32] reported that the amelioration of joint diseases by LA treatment was associated with reduction in oxidative stress, as well as with the inhibition of inflammatory cytokine activation and NF- κ B DNA binding activity. Furthermore, LA-induced decreased intracellular ROS in lymphocytes obtained from the inguinal lymph nodes of arthritic mice and prevented bone destruction *in vivo* and osteoclastogenesis *in vitro*.

Effects of exposure to hyperbaric oxygen

Hyperbaric oxygen therapy leads to new vasoconstriction and hyperoxygenation, making it an effective treatment option for various clinical disorders [33–35]. This treatment involves administration of 100% oxygen concentration at a pressure greater than atmospheric pressure at sea level, usually equivalent to 2–3 ATA. However, a standard procedure of hyperbaric oxygen therapy is known to cause excessive production of ROS in several tissues and organs [33, 34], suggesting that oxidative stress, which is induced by hyperbaric oxygen therapy, accelerates the tissue damage.

We determined that a pressure of 1.25 ATA and an oxygen concentration of 36% are required for obtaining effective responses with regard to oxidative metabolism [7, 8]. In this study, we exposed the rats to moderate atmospheric pressure and oxygen concentration as compared to those exposed to classical hyperbaric oxygen therapy with 100% oxygen concentration at 2 ATA; we observed that these conditions of hyperbaric oxygen exposure inhibit the excessive production of ROS. Furthermore, these conditions of hyperbaric oxygen exposure are more cost-effective and safe; i.e. treatment at these conditions is not associated with the risk of accidents such as eardrum split. We observed that an excessive production of ROS in spontaneously hypertensive rats was inhibited by exposure to hyperbaric oxygen with 36% oxygen concentration at 1.25 ATA [36].

Intraarticular pressure is high in inflammatory joints because of decreased compliance of the joint wall, which is attributable to synovial membrane swelling and capsule fibrosis [37]. Other factors contributing to increased intraarticular pressure include movement and accumulation of synovial fluids in chronically involved joints [10]. Together with the reduced capillary density, this elevated pressure could decrease the rate of capillary blood flow and induce lowering of oxygen tension in synovial fluids and repetitive ischemia-reperfusion injury in the inflammatory joint [11–13]. This suggests that arthritic joints are characterized by hypoxia, which is caused by increased oxygen demand and decreased blood flow induced by increased intraarticular pressure. These observations show that a potential therapeutic approach for treating arthritis would be to enhance the partial pressure of oxygen and increase the levels of dissolved oxygen for the elimination of ROS. An increase

in atmospheric pressure and oxygen concentration enhances the partial pressure of oxygen; this increases the oxygen tension in the affected tissues and the concentration of dissolved oxygen in the plasma, thus enhancing the activity of oxidative enzymes in the mitochondria and, consequently, the rate of oxidative metabolism in cells and tissues. Previously, we reported that the beneficial effects of hyperbaric oxygen exposure with 36% oxygen concentration at 1.25 ATA were associated with an increased oxidative enzyme activity in skeletal muscle fibers and spinal motoneurons in rats [7, 8]. In addition, the possible beneficial effects of exposure to hyperbaric oxygen have been reported; the increase in blood glucose level, which is related to growth, of type 2 diabetic rats has been reported to be inhibited by exposure to hyperbaric oxygen [38–40]. We observed that the levels of dROMs decreased in arthritic rats following exposure to hyperbaric oxygen (Fig. 3A); this indicated that the conditions of hyperbaric oxygen exposure used in this study are effective for decreasing the activity of ROS, which are overproduced in arthritic rats. However, further studies are warranted to elucidate whether these effects of exposure to hyperbaric oxygen persist over prolonged periods. In addition, clinical trials must be performed to evaluate the efficacy of exposure to hyperbaric oxygen for the treatment of arthritic patients.

The arthritis scores did not differ between the arthritic rats that were and were not exposed to hyperbaric oxygen (Fig. 2). This result indicates that the effects of exposure to hyperbaric oxygen on the dROM and CRP levels (Fig. 3) did not correspond with those on the morphological profiles of individual paws in arthritic rats. Morphological effects of exposure to hyperbaric oxygen on rats may be observed if they were treated for a prolonged period; in this study, we

sacrificed the rats only 3 weeks after immunization. Further studies are required to clarify time-dependent effects of exposure to hyperbaric oxygen on the morphological profiles of paws in arthritic rats.

Conclusion

A minor but significant decrease was induced in the serum levels of dROMs and CRP in arthritic rats after exposure to hyperbaric oxygen. We conclude that the conditions of hyperbaric oxygen exposure used in this study are effective to decrease the activity of ROS, which are overproduced at an early stage of arthritis.

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